

**The relative contribution of intramyocellular lipid to whole body fat oxidation is reduced with age, but subsarcolemmal lipid accumulation and insulin resistance are only associated with overweight individuals**

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**ABSTRACT**

Insulin resistance is closely related to intramyocellular lipid (IMCL) accumulation, and both are associated with increasing age. It remains to be determined to what extent perturbations in IMCL metabolism are related to the ageing process *per se*. On two separate occasions whole-body and muscle insulin sensitivity (euglycaemic hyperinsulinaemic clamp with 2-deoxyglucose) and fat utilisation during 1 h of exercise at 50%  $\text{VO}_2\text{max}$  ([U- $^{13}\text{C}$ ]palmitate infusion combined with electron microscopy of IMCL) were determined in young lean (YL), old lean (OL), and old overweight (OO) males. OL displayed comparable IMCL content and insulin sensitivity to YL, whereas OO were markedly insulin resistant and had over 2-fold greater IMCL in the subsarcolemmal (SSL) region. Indeed, whereas the plasma free fatty acid rate of appearance and disappearance was twice that of YL in both OL and OO, SSL only increased during exercise in OO. Thus, skeletal muscle insulin resistance and lipid accumulation often observed in older individuals are likely due to lifestyle factors, rather than inherent ageing of skeletal muscle as usually reported. However, age *per se* appears to cause exacerbated adipose tissue lipolysis, suggesting that strategies to reduce muscle lipid delivery and improve adipose tissue function may be warranted in older overweight individuals.

The global prevalence of type 2 diabetes is most apparent in older people (1), and it is estimated that the number of people over 65 years of age with diabetes will have increased 4.5 fold by 2050 (2). Gaining mechanistic insight of age related insulin resistance and strategies to improve insulin sensitivity with age are clearly warranted. Although ageing is associated with insulin resistance, age *per se* does not appear to cause insulin resistance (3, 4, 5). Several factors that likely contribute to age related insulin resistance include increased abdominal adiposity and reduced physical activity (3, 4), along with declines in muscle mass (6, 7). Of note, intramyocellular lipid (IMCL) accumulates with age, particularly in subsarcolemmal (SSL) regions (8), and has been strongly associated with insulin resistance (9, 10, 11, 12). Indeed, SSL lipid accumulation has been linked to the accumulation of metabolites, such as diacylglycerol (DAG) and ceramide, thought by some (13, 14, 15), but not others (16), to contribute to impaired insulin-stimulated muscle glucose uptake. Nevertheless, it remains contentious as to which factors associated with age influence IMCL accumulation.

The accumulation of IMCL and associated metabolites likely result from an imbalance between muscle lipid delivery and oxidation. Indeed, studies have demonstrated reduced free fatty acid (FFA) oxidation in older people compared to young, despite whole-body lipolysis and plasma FFA availability being greater at rest and during exercise at the same absolute and relative intensities (17, 18). Linked to this, several studies have suggested age related blunting of FFA oxidation and increased IMCL accumulation are a result of reduced muscle mitochondrial content (8) and function (3, 19, 20) with age. However, increased adiposity and reduced habitual levels of physical activity also affect FFA flux and oxidation in older individuals (21), and studies to date have not controlled for these factors when investigating changes in muscle IMCL metabolism with age. Therefore, we investigated the effect of

ageing on whole-body and skeletal muscle lipid metabolism, with parallel characterization of muscle insulin sensitivity, in lean young and older individuals matched for estimated habitual physical activity levels and body composition. To determine the effect of adiposity and reduced physical activity on the ageing process, the older lean individuals were also compared to a group of older overweight individuals matched for lean mass. We hypothesized that an age-associated imbalance between FFA delivery and oxidation in skeletal muscle during exercise would only be observed in older overweight individuals, which would manifest as reduced IMCL oxidation and increased IMCL storage, particularly in the SSL region, and be associated with skeletal muscle insulin resistance.

## RESEARCH DESIGN AND METHODS

### Subjects

Seven young lean, (YL; BMI  $<25 \text{ kg/m}^2$ ), old lean (OL; body mass index (BMI)  $<25 \text{ kg/m}^2$ ), and old overweight (OO; BMI  $>27 \text{ kg/m}^2$ ) healthy, recreationally active male volunteers participated in the present study, which was approved by the University of Nottingham's Medical School Ethics Committee in accordance with the Declaration of Helsinki. Before taking part, all subjects underwent routine medical screening and completed a quality of life (SF-36) questionnaire indicating their ability to perform physical activity. They also completed a general health questionnaire indicating their habitual frequency of performing moderate to high intensity physical activities including team sports, resistance exercise, running, cycling, and swimming (Table 1). Informed consent was obtained from all volunteers before participating in the study and they were made aware that they were free to withdraw at any point. On a separate visit all subjects performed an incremental exhaustive exercise test on an electronic-braked cycle ergometer (Excalibur, Lode, The Netherlands) to determine their maximal rate of oxygen consumption ( $\text{VO}_{2\text{max}}$ ; Quark CPET, Cosmed, Italy) and the workload that would elicit 50%  $\text{VO}_{2\text{max}}$ , which was confirmed in a familiarization visit at least 3 days later.

### Experimental Protocol

Subjects attended the laboratory on two occasions separated by at least 1 week. On the first occasion they arrived at 0800 after an overnight fast, having abstained from exercise and alcohol for the previous 48 hours, in order to determine their body composition and insulin sensitivity. Trunk, leg and arm composition using standardized regions were analyzed by a single operator using dual energy x-ray absorptiometry (DEXA; Lunar Prodigy, GE

Healthcare, US). Subjects then rested semi-supine on a bed and underwent a 3 h euglycaemic (4.5 mmol/L) hyperinsulinaemic ( $60 \text{ mU} \cdot \text{m}^{-2} \cdot \text{min}^{-1}$ ) clamp (22) in combination with the intravenous infusion of 2-deoxy-D-glucose (2DG;  $6 \text{ mg} \cdot \text{kg}^{-1} \cdot \text{h}^{-1}$ ) to assess whole body and skeletal muscle insulin sensitivity, respectively. 2DG is a glucose analogue that closely resembles glucose in the characteristics of its transport but is metabolized by muscle to the 6-phosphate derivative (2DG6P). Thus, muscle 2DG6P is effectively trapped and its content can be determined as a direct measure of muscle glucose uptake (23). Indirect calorimetry (GEMNutrition Ltd, UK) was performed prior to and after 2 h of the clamp.

On the second visit, volunteers again reported to the laboratory following an overnight fast and rested semi-supine on a bed for infusion of  $\text{NaH}^{13}\text{CO}_3$  (Cambridge Isotope Laboratories, USA) and  $[\text{U}-^{13}\text{C}]$ palmitate (99% enriched; Cambridge Isotope Laboratories, USA) bound to 4.5% human serum albumin (Zenalb 4.5, Bio Products Laboratory Limited, UK) at a ratio of approximately 3:1 ( $1.94:0.64 \text{ } \mu\text{mol/L}$ ). Following a  $63.75 \text{ } \mu\text{g/kg}$  bolus of  $\text{NaH}^{13}\text{CO}_3$  to prime the bicarbonate pool (24),  $[\text{U}-^{13}\text{C}]$ palmitate was infused at a rate of  $0.19 \text{ mg} \cdot \text{kg}^{-1} \cdot \text{h}^{-1}$  for 2 h, which then increased to  $0.28 \text{ mg} \cdot \text{kg}^{-1} \cdot \text{h}^{-1}$  at the onset of 1 h cycling exercise at 50%  $\text{VO}_{2\text{max}}$ .

### **Sample collection and analysis**

During the first visit arterialized-venous blood (25) was obtained before and every 5 min throughout the clamp for measurement of blood glucose concentration (Stat Analyzer, YSI Inc, USA) and every 30 min throughout the clamp for subsequent analysis of serum insulin using a solid-phase  $^{125}\text{I}$  radioimmunoassay kit (Human Insulin Assay, Merck Millipore, USA), and plasma 2DG via gas-chromatography mass-spectrometry (GC-MS; MD800, Fisons, UK; 23). Needle biopsy samples were obtained from the vastus lateralis (26) before and immediately after the clamp and snap frozen in liquid nitrogen. At a later date, 30 mg of

wet muscle was pulverized for analysis of 2DG6P content using a commercial spectrophotometric kit method (Cosmo Bio Ltd, Japan; 27). In addition, approximately 20 mg of wet muscle from the baseline biopsy was used to determine muscle citrate synthase (CS) maximal activity spectrophotometrically (28) and carnitine palmitoyltransferase 1 (CPT1) maximal activity using a radioisotope assay (29). Total RNA was also extracted from approximately 20 mg of wet muscle tissue (Trizol reagent; Invitrogen Ltd, UK) and following generation of first-strand cDNA (SuperScript III kit; Invitrogen Ltd, UK), the relative abundance of mRNA of 12 genes from pathways involved in FFA oxidation and IMCL metabolism was determined using RT-PCR microfluidic cards (Applied Biosystems, USA, 29).

On the second experimental visit, blood samples were obtained before and every 10 min during exercise, analyzed immediately for blood lactate concentration (2300 Stat Analyzer; YSI Inc, USA) and, following centrifugation, plasma was stored at -80°C. Plasma treated with tetrahydrolipostatin (30 µg/mL plasma) was analyzed for total FFA (NEFA C kit, WAKO Chemicals, Germany) on an automated analyzer (ABX Pentra 400, Horiba Medical Ltd., France). Plasma separated from EGTA treated blood was analyzed for [U-<sup>13</sup>C]palmitate and palmitate by TSQ triple quadrupole gas-chromatography-mass-spectrometry/mass spectrometry (GC-MS/MS, Thermo, UK) and GC-MS (MD800, Fisons, UK ) respectively, after addition of a heptadecanoic internal standard and derivatization to their methyl esters (30). High-performance liquid-chromatography (HPLC) with electrochemical detection was used to measure plasma epinephrine and norepinephrine concentrations (31). Breath samples were also collected every 10 min during exercise via one-way valve bags and introduced into vacuumed glass tubes (Exetainer, Labco Ltd, UK) for subsequent <sup>13</sup>CO<sub>2</sub> enrichment analysis by continuous-flow isotope-ratio MS (CF-IRMS; AP2003 Breath Gas System, Analytical

Precision, UK; 32). During the last 10 min of exercise when the  $^{13}\text{CO}_2$  production was at a steady-state and therefore no longer being retained by the muscle (negating the requirement for an acetate recovery factor), indirect calorimetry was performed (Quark CPET system, Cosmed, Italy). In addition, a vastus lateralis needle biopsy (26) was obtained immediately before and after the exercise bout and processed within 10 seconds to minimise *ex vivo* changes in intracellular metabolism and contamination of the IMCL pool by extracellular adipocytes. A 5 mg portion buffered in ice-cold 3% glutaraldehyde/0.1 M sodium cacodylate (pH 7.4) and stored at 4°C for subsequent electron microscopy processing, and the remainder immediately frozen in lipid nitrogen. Samples for transmission electron microscopy were fixed in 1% osmium tetroxide, dehydrated in graded ethanol series and embedded in two resin blocks. Three ultrathin 70-90 nanometer sections were cut from each block, mounted on copper grids, and stained in uranyl acetate and lead acetate, with one section randomly selected to be visualized at x4200 magnification. Approximately 40 fields of view from up to 40 longitudinal fibres were systematically randomly selected by a blinded operator using the corners of copper grid squares as a guide. This method obtained at least 6 images per sample containing a SSL region, which was required for reproducible estimation of IMCL droplet (LD) characteristics. Images were analyzed using Image J to determine percentage of intermyofibrillar (IMF) and SSL area covered by LD, LD size, and total number of LD per square micrometer of local tissue area, which have been previously shown (8) to produce values similar to 3D stereology volume estimates (33). In addition, a portion of the pre-exercise biopsy was freeze-dried, dissected free of visible blood and connective tissue, pulverized and used for the quantification of DAG and ceramide. Briefly, 50 ng internal standard (1,3[d5]-15:0 DAG) was added to 5 mg muscle, from which total muscle lipids were extracted in  $\text{CHCl}_3$ :MeOH:H<sub>2</sub>O and the most abundant DAG (diC16:0, C16:0/C18:1, diC18:1) and ceramide (C16:0, C18:0, C18:1, C20:0, C24:1, C24:0) species were quantified



by LC-MS-MS (Quattro Ultima, Micromass Ltd, UK; 34, 35). Peak areas were normalised to the internal standard and converted to absolute concentrations using a standard curve specific for each species. A further portion of muscle powder was also used for the determination of muscle creatine, phosphocreatine, glycogen, lactate, and acetylcarnitine as previously described (36).

### Calculations

Insulin sensitivity index ( $SI_{Clamp}$ ) was calculated using the equation of Matsuda and DeFronzo ( $SI_{Clamp} = M/(G \times \Delta I)$ ; 22) where steady-state (120-180 min) glucose disposal (M) is normalized for steady-state blood glucose concentration (G; mmol/L) and the difference between fasting and steady-state plasma insulin concentrations ( $\Delta I$ ; mU/L). Indirect calorimetry calculations both at rest and during exercise were performed according to non-protein stoichiometric equations (37) and normalised to lean body mass (DEXA). The rate of appearance ( $R_a$ ), disappearance ( $R_d$ ), and oxidation of palmitate during the final 10 min of exercise were used to calculate total plasma FFA kinetics by dividing the fractional contribution of plasma palmitate to total plasma FFA concentration as previously described (38). The contribution of other fat sources was calculated by subtracting plasma FFA oxidation from total fat oxidation calculated via indirect calorimetry.

### Statistical Analysis

Differences between groups at baseline, and within and between groups during exercise, were analyzed using a one- and two-way ANOVA, respectively (GraphPad Prism 6.0, GraphPad Software Inc, USA). When a significant main effect was observed, Tukey's and Sidak's post-hoc test was performed, respectively, to identify individual differences. Statistical significance was set at  $P < 0.05$ , and all values are presented as means  $\pm$  SEM.

## RESULTS

### Subject characteristics

In line with the inclusion criteria OL and YL had a similar body composition. However, OO had greater trunk, arm and leg fat masses compared with OL and YL subjects, but similar whole-body, arm and leg fat free mass (Table 1). Furthermore, self-reported levels of habitual physical activity were similar between OL and YL, but less in OO compared to OL. Both OL and OO had similar absolute ( $\text{mL}/\text{min}$ ) and relative ( $\text{mL}\cdot\text{kg lbm}^{-1}\cdot\text{min}^{-1}$ )  $\text{VO}_2\text{max}$  but these were less than in YL, as were the corresponding absolute workload and heart rate at 50%  $\text{VO}_2\text{max}$ .

### Skeletal muscle insulin sensitivity and lipid metabolite content

Steady-state serum insulin and glucose disposal during the euglycaemic hyperinsulinaemic clamp for OL, YL, and OO were ( $119.6 \pm 7.0$ ,  $117.7 \pm 7.8$ , and  $137.5 \pm 4.3$   $\text{mU}/\text{L}$ ) and ( $57.8 \pm 5.6$ ,  $65.1 \pm 5.6$ , and  $41.6 \pm 5.2$   $\mu\text{mol}\cdot\text{kg lbm}^{-1}\cdot\text{min}^{-1}$ ;  $P<0.01$  OL and YL vs. OO), respectively. As such, OL and YL had similar  $\text{SI}_{\text{Clamp}}$  that were 57% ( $P<0.05$ ) and 86% ( $P<0.01$ ) greater than OO, respectively (Figure 1A). Furthermore, muscle 2DG6P accumulation during the clamp was not different between OL and YL, but was less than half that of YL in OO ( $P<0.01$ ; Figure 1B), and OL and YL had similar steady-state plasma 2DG concentrations during the clamp, which were less than OO ( $50.0 \pm 1.9$  and  $48.8 \pm 2.2$  vs.  $72.6 \pm 2.2$   $\mu\text{mol}/\text{L}$ , respectively;  $P<0.05$ ; Figure 1C). Insulin-stimulated resting energy expenditure increased by more than 10% in both OL ( $P<0.01$ ) and YL ( $P<0.05$ ), but did not change in OO ( $P=1.0$ ; Figure 1D). There were no differences in muscle DAG species between groups with the exception of diC18:1, which was lower in OL and YL compared to

OO (both  $P < 0.05$ ; Figure 2A). Similarly most muscle ceramide species did not differ except C20:0, which was lower in YL compared to OO ( $P < 0.01$ ; Figure 2B).

### **Whole body substrate metabolism during exercise**

Whole body energy expenditure during the last 10 min of 1 h of exercise at 50%  $\text{VO}_{2\text{max}}$  was lower in OL and OO compared to YL (both  $P < 0.05$ ; Figure 3A), but the relative contribution from total fat oxidation to energy expenditure was similar ( $42.4 \pm 3.1$ ,  $40.1 \pm 4.6$  and  $43.9 \pm 6.5\%$ , respectively). Nevertheless, the oxidation of fat from sources other than plasma FFA (i.e. predominantly from IMCL) was almost 3-fold lower in OL and OO compared to YL (both  $P < 0.05$ ; Figure 3A), such that the relative contribution of these sources to total fat oxidation was around half that of YL ( $38.7 \pm 7.7$  and  $45.0 \pm 7.9$  vs.  $71.9 \pm 3.1\%$ , respectively;  $P < 0.01$ ; Figure 3A). Plasma FFA Ra was similar between OL and OO ( $24.2 \pm 2.9$  vs.  $24.3 \pm 5.3 \mu\text{mol} \cdot \text{kg lbm}^{-1} \cdot \text{min}^{-1}$ ), and greater compared to YL ( $13.8 \pm 2.3 \mu\text{mol} \cdot \text{kg lbm}^{-1} \cdot \text{min}^{-1}$ ;  $P < 0.05$ ), but there were no differences in plasma FFA concentration ( $0.62 \pm 0.06$ ,  $0.58 \pm 0.09$ , and  $0.45 \pm 0.08$  for OL, OO and YL, respectively). Plasma FFA Rd was also similar between OL and OO, but greater in OL compared to YL ( $P < 0.05$ ; Figure 3B). In contrast, whereas the percentage of plasma FFA Rd oxidized was similar between OL and YL ( $54.4 \pm 5.9$  and  $52.7 \pm 3.4\%$ , respectively; Figure 3C), it was lower in OO ( $42.2 \pm 1.2\%$ ) compared to OL ( $P < 0.05$ ) and YL ( $P = 0.07$ ; Figure 3C).

From similar baseline concentrations, plasma norepinephrine increased to a similar steady-state in OL and OO throughout 1 h of exercise, and was around 1.5-fold greater than the steady-state concentration achieved in YL (both  $P < 0.05$  respectively; Figure 3D). However, there were no differences between groups in baseline or steady-state plasma epinephrine ( $0.25 \pm 0.02$  to  $0.53 \pm 0.11$ ,  $0.25 \pm 0.06$  to  $0.34 \pm 0.06$ , and  $0.24 \pm 0.04$  to  $0.43 \pm 0.06$

nmol/L) or blood lactate ( $0.71 \pm 0.06$  to  $1.50 \pm 0.29$ ,  $0.97 \pm 0.13$  to  $2.05 \pm 0.31$ , and  $0.87 \pm 0.09$  to  $1.73 \pm 0.28$  mmol/L) concentrations in OL, OO and YL, respectively.

### **IMCL and skeletal muscle oxidative metabolism during exercise**

The area of SSL region covered by LD was similar between OL and YL at rest and did not change during exercise (Figure 4A). However, SSL area covered by LD in OO was almost 3-fold greater at rest compared with YL ( $P < 0.05$ ) and increased during exercise ( $P < 0.05$ ), such that post-exercise it was greater than both OL ( $P < 0.05$ ) and YL ( $P < 0.01$ ; Figure 4A). This was predominantly due to a 25% increase in average SSL LD size in OO ( $P = 0.05$ ; Figure 4B). In contrast, exercise caused a decrease ( $P < 0.01$ ) in both the number of IMF LD ( $0.024 \pm 0.001$  to  $0.017 \pm 0.003$ ,  $0.022 \pm 0.003$  to  $0.015 \pm 0.002$ , and  $0.023 \pm 0.03$  to  $0.018 \pm 0.003$  LD/ $\mu\text{m}^2$  for OL, YL and OO, respectively) and area covered by LD (Figure 4C). The latter was isolated to a 40% reduction in IMF area covered by LD in YL (0.05; Figure 4C). Average IMF LD size was 45% greater in OO compared to OL and YL post-exercise (both  $P < 0.01$ ; Figure 4D).

Resting skeletal muscle glycogen (Figure 5A), phosphocreatine (Figure 5B), and lactate (Figure 5C) content was similar between OL and YL and did not change measurably during exercise, whereas acetylcarnitine content increased during exercise by around 7 ( $P < 0.001$ ) and 3 fold ( $P < 0.05$ ), respectively. However, resting muscle glycogen and phosphocreatine content were lower ( $P < 0.05$ ) and muscle lactate content more than doubled during exercise ( $P < 0.05$ ) in OO. Nevertheless, there were no significant differences in maximal CS ( $116.8 \pm 12.6$ ,  $94.7 \pm 8.8$ , and  $84.1 \pm 8.3$  nmol·mg protein<sup>-1</sup>min<sup>-1</sup>, respectively) or CPT1 ( $2.3 \pm 0.3$ ,  $1.8 \pm 0.3$ , and  $2.0 \pm 0.1$  nmol·mg protein<sup>-1</sup>min<sup>-1</sup>, respectively) activities between OL, YL and OO, respectively, although the former tended to be greater in OL vs. OO ( $P = 0.08$ ).

**Skeletal muscle gene expression**

The relative expression of 12 skeletal muscle transcripts involved in fatty oxidation and IMCL turnover are presented in Table 2. HADHB and PLIN2 expression were greater in OL compared to YL ( $P<0.05$ ), whereas ACACB, SPTLC1, and DGKD expression were lower in YL compared to OO (all  $P<0.05$ ). Furthermore, PLIN2 gene expression was greater in OL vs. OO ( $P<0.05$ ) respectively.

## DISCUSSION

Insulin resistance is closely related to IMCL accumulation, and both are associated with increasing age. However, it remains to be determined to what extent perturbations in IMCL metabolism are related to the ageing process *per se* or secondary to age-related changes in lifestyle. Thus, by matching young and older volunteers for body composition and self-reported habitual physical activity levels the present study demonstrated that lean older individuals display comparable IMCL content and insulin sensitivity to their younger counterparts. On the other hand, ageing *per se* appeared to cause an exacerbated lipolytic response to exercise due, at least in part, to an increased sympathetic response. Coupled with increased adiposity and reduced habitual physical activity levels in an age-matched group this resulted in SSL IMCL accumulation, and may mechanistically help explain the association between increased IMCL and skeletal muscle insulin resistance in older individuals.

In line with several studies that suggest ageing *per se* does not cause insulin resistance (3, 4, 5), there was no difference in whole-body glucose disposal, skeletal muscle 2DG6P accumulation, or the energy expenditure response during a euglycaemic hyperinsulinaemic clamp between old and young individuals matched for body composition and self-reported physical activity in the present study. Furthermore, the finding that whole-body and skeletal muscle insulin action was reduced in old overweight individuals with a similar lean body mass but lower self-reported physical activity supports the notion that lifestyle factors are more influential in the development of age-related insulin resistance (3, 4, 5, 39, 40, 41). A possible link between these factors and reduced skeletal muscle insulin sensitivity is the accumulation of SSL IMCL (9, 10) and associated lipid metabolites such as DAG and ceramide (13, 14, 15). Indeed, whereas there was no difference in SSL IMCL between lean

old and young, SSL IMCL was more than 2-fold higher in the older overweight individuals, which is in agreement with a 2 and 3-fold greater content observed in lean sedentary older individuals (8) and type 2 diabetes (9), respectively. However, although the skeletal muscle content of the predominant DAG and ceramide species were not different between lean old and young they were also not greater in old overweight, with the exception of diC18:1 DAG and C20:0 ceramide. Indeed, total muscle DAG and ceramide do not correlate well with insulin sensitivity but specific lipid species, particularly sarcolemmal saturated DAG, may influence insulin action (42).

Why IMCL accumulates, particularly in the SSL region, is not clear, but several studies have demonstrated reduced FFA oxidation in older individuals despite increased whole body lipolysis and FFA availability compared to young at rest and during exercise (17, 18). Indeed, although the relative contribution of fat oxidation to total energy expenditure during exercise was not different between the young and old groups of the present study, there was an elevated plasma norepinephrine, FFA Ra, and FFA Rd response to exercise at the same relative intensity observed in both the lean and overweight older individuals, suggesting an effect of age *per se* on whole body responses. This would fit with previous reports that age associated increments in norepinephrine are independent of habitual physical activity and likely due increased sympathetic activity rather than reduced norepinephrine clearance (43). As a consequence, the relative contribution of IMCL to fat oxidation was reduced in both old lean and overweight compared to young individuals. This is remarkable given there was presumably a greater lipolytic stimulus to IMCL by norepinephrine in the older individuals (44, 45), and would suggest a potent inhibitory effect of plasma derived FFA or a blunted contraction induced IMCL hydrolysis. Furthermore, a novel finding of the present study was that, assuming similar rates of adipose tissue FFA re-esterification (where FFA released from

adipose tissue is reincorporated in a futile cycle), lean older individuals were able to oxidise a larger proportion of the excess FFA delivered during exercise compared to the older overweight individuals, where it deposited in SSL lipid droplets. This not only suggests that a more general, chronic imbalance between skeletal muscle FFA delivery and oxidation may contribute to IMCL accumulation, but also provides evidence for distinct roles of the localised IMCL pools. For example, the reduction in the number of IMF lipid droplets during exercise in the younger individuals suggests that this pool is used for muscle contraction, possibly in an 'all or nothing' fashion, whereas the deposition in the SSL pool suggests a role in buffering/trafficking of FFA influx (46), and perhaps insulin resistance. Interestingly, an improvement in insulin sensitivity has been previously observed with reduced SSL but not IMF IMCL following 10 to 12 weeks of exercise training where the capacity to oxidise FFA was increased (9, 10).

In addition to reduced energy expenditure, such as observed during insulin-stimulated conditions of the present study, several mechanisms may explain the apparent inability of skeletal muscle of older overweight men to oxidize excess FFA delivery. For example, it has been suggested that aging is associated with impaired *in vivo* (19) and *in vitro* (3, 20) skeletal muscle mitochondrial ATP production, as well as a reduction in mitochondrial content (8), independently of adiposity. However, there was no difference in skeletal muscle maximal CS activity, maximal and relative CPT1 activity, or phosphocreatine, glycogen, lactate, and acetylcarnitine metabolism during exercise between the lean old and young participants in the present study, all of which are markers of *in vivo* muscle oxidative capacity. On the other hand, the disparity between old lean and old overweight participants in the ability to oxidize excess fatty acids may be due to differences in partitioning of skeletal muscle lipid and a diversion of fatty acids from oxidation towards synthesis of IMCL and other lipid species.



For example, older lean individuals had a greater mRNA expression of perilipin 2 (PLIN2), a lipid droplet bound protein involved IMCL hydrolysis, and  $\beta$ -hydroxyacyl-CoA dehydrogenase (HADHB), an intramitochondrial enzyme that catalyses a rate-limiting step in  $\beta$ -oxidation, whereas old overweight had greater mRNA expression of acetyl-CoA carboxylase 2 (ACACB), which produces malonyl-CoA and inhibits CPT1, the rate limiting step for fatty acid entry into mitochondria. The PLIN2 expression in particular would fit previous reports in overweight individuals of impaired IMCL turnover and FFA release toward mitochondrial oxidation (44, 47). Furthermore, old overweight had a greater expression of diacylglycerol kinase delta (DGKD), which phosphorylates diacylglycerol to produce phosphatidic acid, and serine palmitoyltransferase (SPTLC1), a rate-limiting step in ceramide synthesis, compared to young lean individuals. Both of these observations fit with the greater muscle content of some of the DAG and ceramide species in the present study. A similar gene expression pattern has also been previously observed in insulin resistant individuals (48), but how this translates into protein content/activity and whether it is cause or effect requires further investigation.

In conclusion, it is our assertion that increased IMCL (4, 8, 19) and reduced insulin sensitivity, mitochondrial capacity, and fat oxidation (3, 17, 18, 19, 20, 21, 49) often observed in older individuals are likely due to lifestyle factors rather than aging *per se* as commonly reported. However, age *per se* appears to increase the systemic sympathetic response to exercise and cause exacerbated adipose tissue lipolysis. Compounded by greater adiposity, the increased FA delivery appears to cause SSL IMCL accumulation in physically inactive older individuals. Thus, targeted strategies to reduce muscle lipid delivery and improve adipose tissue function may be warranted, particularly as physical inactivity appears to worsen the inability to suppress adipose tissue lipolysis in older individuals (50).

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TABLES

**Table 1.** Characteristics of young lean (YL), old lean (OL), and old overweight (OO) male participants.

	YL	OL	OO
n	7	7	7
Age (y)	21.5 ± 1.0	69.7 ± 0.9 <sup>+++</sup>	68.6 ± 0.8 <sup>***</sup>
Statin use (n)	0	3	4
Body mass (kg)	71.8 ± 3.6	70.3 ± 2.4	86.3 ± 1.8 <sup>***,†††</sup>
BMI (kg/m <sup>2</sup> )	22.4 ± 0.7	24.0 ± 0.6	29.0 ± 0.7 <sup>***,†††</sup>
Lean mass (lbm; kg)	55.9 ± 3.2	51.3 ± 1.5	55.6 ± 1.9
Arm lean mass (kg)	7.6 ± 0.4	6.7 ± 0.3	7.4 ± 0.3
Leg lean mass (kg)	21.8 ± 0.4	18.3 ± 0.6	19.6 ± 0.7
Trunk fat mass (kg)	4.6 ± 0.8	7.2 ± 1.1	16.0 ± 0.8 <sup>***,†††</sup>
Arm fat (kg)	0.9 ± 0.1	1.5 ± 0.2	2.6 ± 0.3 <sup>***,†††</sup>
Leg fat (kg)	4.7 ± 0.4	4.9 ± 0.4	7.4 ± 0.6 <sup>†</sup>
Fasting blood glucose (mmol/L)	4.5 ± 0.1	4.7 ± 0.1	5.0 ± 0.1 <sup>*</sup>
Fasting serum insulin (mU/L)	10.6 ± 1.4	7.4 ± 1.6	12.6 ± 1.2 <sup>†</sup>
HOMA IR	2.14 ± 0.32	1.60 ± 0.36	2.81 ± 0.25 <sup>†††</sup>
Physical activity frequency (occasions/week)	3.5 ± 0.5	5.1 ± 1.2	1.8 ± 0.7 <sup>†</sup>
VO <sub>2</sub> max (L/min)	3.19 ± 0.19	2.26 ± 0.15 <sup>+++</sup>	2.19 ± 0.13 <sup>***</sup>
VO <sub>2</sub> max (mL·kg lbm <sup>-1</sup> ·min <sup>-1</sup> )	57.4 ± 2.4	44.6 ± 1.9 <sup>+++</sup>	39.9 ± 1.5 <sup>***</sup>
Workload at 50% VO <sub>2</sub> max (W)	93.0 ± 5.9	55.9 ± 5.8 <sup>+++</sup>	46.3 ± 6.7 <sup>***</sup>
Heart rate at 50% VO <sub>2</sub> max (beats/min)	137 ± 2	102 ± 7 <sup>+++</sup>	102 ± 6 <sup>***</sup>

All values (n=7) are means ± standard error of the mean (SEM). \*\*\*P<0.001, OO different to corresponding YL value. †P<0.05, †††P<0.001, OO different to corresponding OL value. +++P<0.001, OL different to corresponding YL value.

**Table 2.** Expression of skeletal muscle transcripts encoding proteins involved in fatty acid oxidation and IMCL in young lean (YL), old lean (OL), and old overweight (OO) males.

	Gene	YL	OL	OO
Fatty acid oxidation	ACACB	0.79 ± 0.04	0.83 ± 0.24	1.38 ± 0.12 <sup>*†</sup>
	CPT1B	1.01 ± 0.25	3.38 ± 1.38	1.76 ± 0.44
	CPT2	0.83 ± 0.07	0.90 ± 0.18	1.02 ± 0.17
	HADHB	0.70 ± 0.07	1.33 ± 0.22 <sup>+</sup>	0.83 ± 0.14 <sup>†</sup>
	ACADM	0.57 ± 0.08	0.73 ± 0.04	0.63 ± 0.08
	ACAT1	0.55 ± 0.09	0.74 ± 0.10	0.66 ± 0.09
IMCL turnover	SPTLC1	0.81 ± 0.09	1.19 ± 0.21	1.29 ± 0.12 <sup>*</sup>
	DGKD	1.05 ± 0.11	1.15 ± 0.24	1.59 ± 0.13 <sup>*</sup>
	DGAT1	0.87 ± 0.06	1.00 ± 0.13	1.12 ± 0.08
	PLIN2	1.15 ± 0.10	1.98 ± 0.30 <sup>+</sup>	1.24 ± 0.16 <sup>†</sup>
	PLIN5	0.93 ± 0.22	1.24 ± 0.26	1.24 ± 0.28
	PNPLA2	0.74 ± 0.07	0.63 ± 0.16	0.62 ± 0.08

All values (n=7) are means ± standard error of the mean (SEM) and expressed as relative mRNA abundance compared to a YL comparator. \*P<0.05, OO different to corresponding YL value. <sup>†</sup>P<0.05, OO different to corresponding OL value. <sup>+</sup>P<0.05, OL different to corresponding YL value.



## FIGURE LEGENDS

**Figure 1.** Insulin sensitivity index (A), skeletal muscle 2-deoxyglucose-6-phosphate accumulation (B), plasma 2-deoxyglucose concentration (C), and whole-body energy expenditure (D) during a 3 h hyperinsulinaemic ( $60\text{mU m}^{-2} \text{min}^{-1}$ ) euglycaemic clamp in young lean (YL, white squares), old lean (OL, black circles), and old overweight (OO, white circles) males. Values represent mean  $\pm$  SEM (n=7). \* $P<0.05$ , \*\*  $P<0.01$ , OO different to corresponding YL value.  $^{\dagger}P<0.05$ ,  $^{\dagger\dagger}P<0.01$ , OO different to corresponding OL value.  $^{\wedge}P<0.05$ ,  $^{\wedge\wedge}P<0.01$ , different to corresponding baseline value.

**Figure 2.** Fasting skeletal muscle diacylglycerol (A) and ceramide content (B) in young lean (white bars), old lean (black bars), and old overweight (hatched bars) males. Values represent mean  $\pm$  SEM (n=7). \* $P<0.05$ , old overweight different to corresponding young lean value.  $^{\dagger}P<0.05$ , old overweight different to corresponding old lean value.

**Figure 3.** Whole-body energy expenditure (A), plasma FFA rate of disappearance (Rd; B), percentage of plasma FFA Rd oxidised (C), and plasma noradrenaline concentration (D) during 1 h of cycling exercise at 50%  $\text{VO}_2\text{max}$  in young lean (YL, white squares), old lean (OL, black circles), and old overweight (OO, white circles) males. The contribution of IMCL to whole-body energy expenditure in 3A assumes non-plasma FFA oxidation is predominantly IMCL. Values represent mean  $\pm$  SEM (n=7). \* $P<0.05$ , \*\*  $P<0.01$ , OO different to corresponding YL value.  $^+P<0.05$ ,  $^{++}P<0.01$ , OL different to corresponding YL value.

**Figure 4.** Percentage area of subsarcolemmal (SSL) region covered by lipid droplets (LD; A), average SSL LD size (B), percentage area of intermyofibrillar (IMF) region covered by LD (C), and average IMF LD size (D) from electron micrographs of skeletal muscle samples taken before (pre exercise) and after (post exercise) 1 h of cycling exercise at 50%  $\text{VO}_2\text{max}$  in young lean (white bars), old lean (black bars), and old overweight (hatched bars) males. Values represent mean  $\pm$  SEM (n=7). \* $P<0.05$ , \*\*  $P<0.01$ , old overweight different to corresponding young lean value.  $^{\dagger}P<0.05$ ,  $^{\dagger\dagger}P<0.01$ , old overweight different to corresponding old lean value.  $^{\wedge}P<0.05$ , different to corresponding pre exercise value.

**Figure 5.** Skeletal muscle glycogen (A), phosphocreatine (B), lactate (C), and acetylcarnitine (D) content before (pre exercise) and after (post exercise) 1 h of cycling exercise at 50%  $\text{VO}_2\text{max}$  in young lean (white bars), old lean (black bars), and old overweight (hatched bars) males. Values represent mean  $\pm$  SEM (n=7). \* $P<0.05$ , old overweight different to corresponding young lean value.  $^{\dagger}P<0.05$ , old overweight different to corresponding old lean value.  $^{\wedge}P<0.05$ ,  $^{\wedge\wedge}P<0.001$ , different to corresponding pre exercise value.

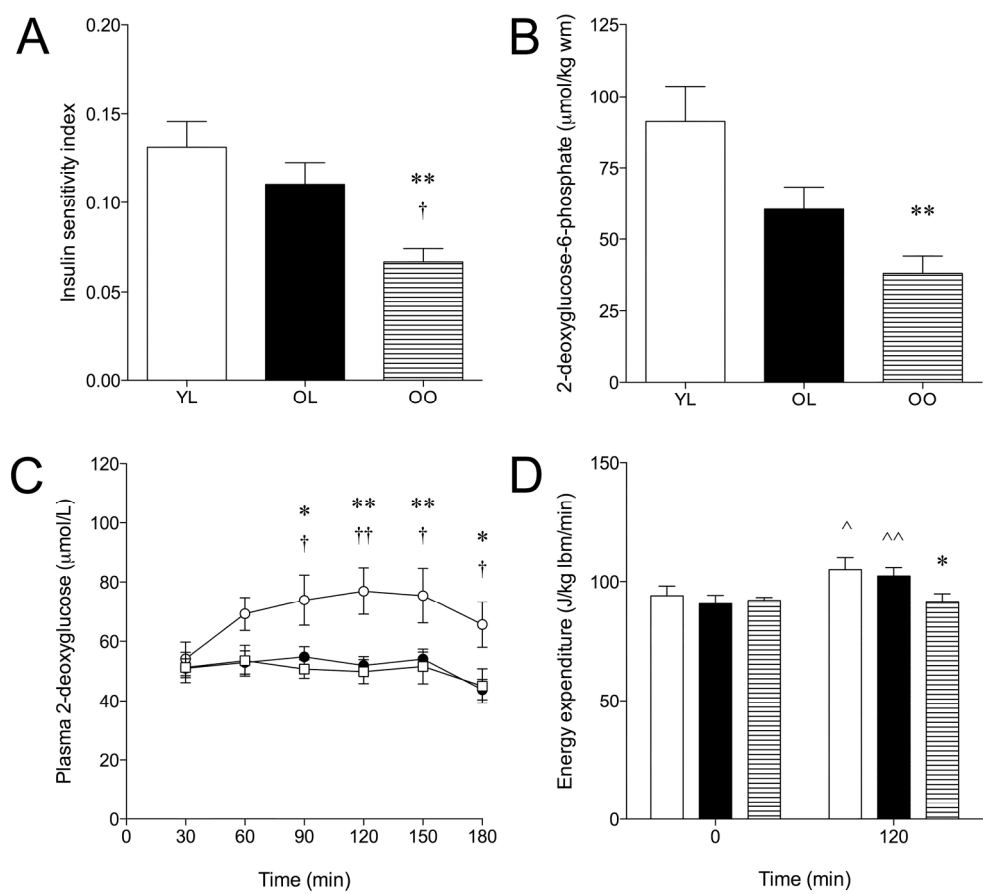


Figure 1  
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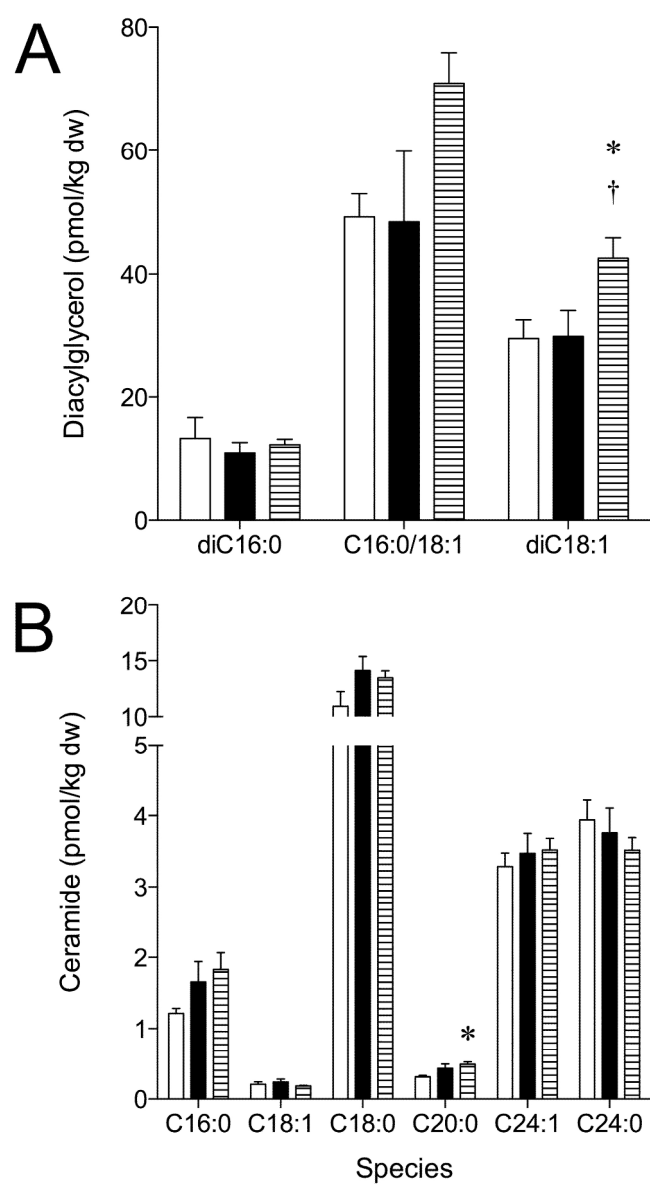


Figure 2  
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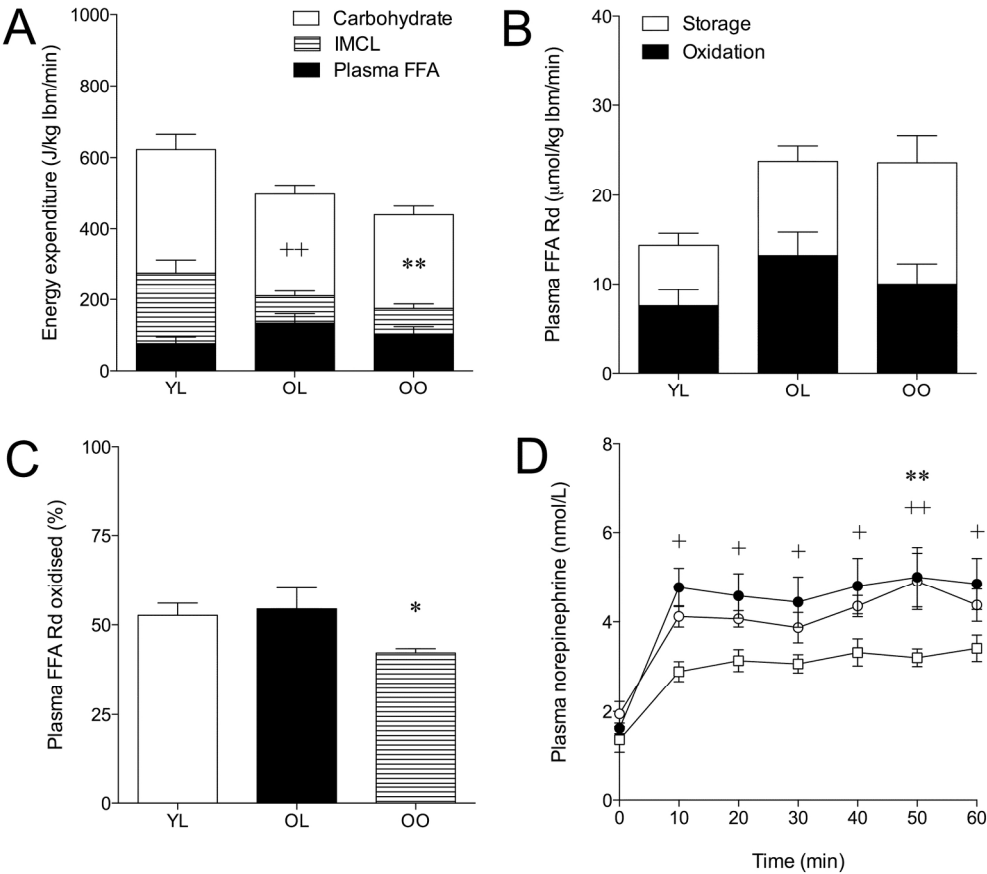


Figure 3  
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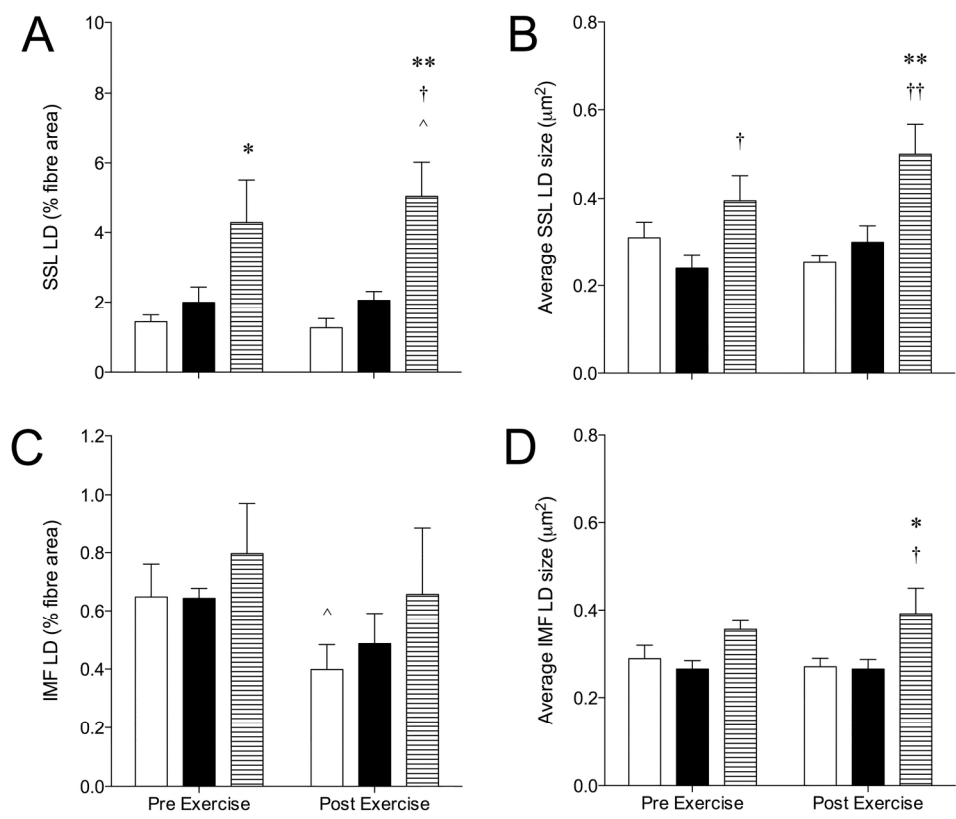


Figure 4  
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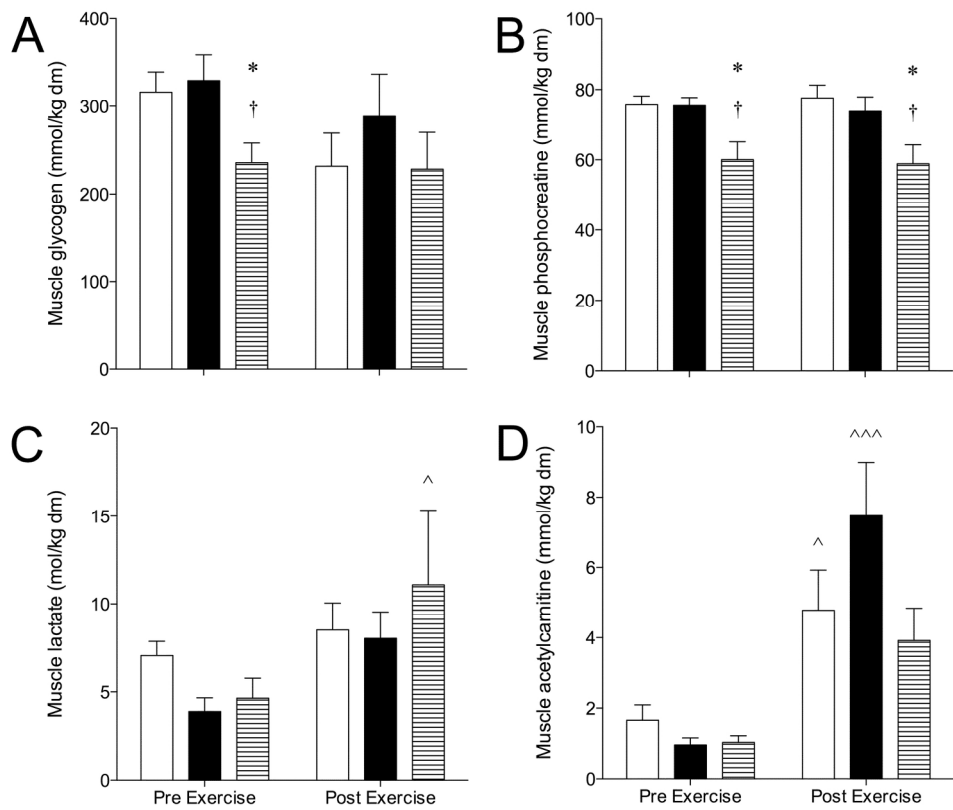


Figure 5  
178x147mm (300 x 300 DPI)